Journal of Neuroimmunology 222 (2010) 76–81

Contents lists available at ScienceDirect

Journal of Neuroimmunology

journal homepage: www.elsevier.com/locate/jneuroim

CSF chemokine alterations related to the clinical course of amyotrophic lateral sclerosis

Takahisa Tateishi, Ryo Yamasaki, Masahito Tanaka, Takuya Matsushita, Hitoshi Kikuchi, Noriko Isobe, Yasumasa Ohyagi, Jun-ichi Kira *

Department of Neurology, Neurological Institute, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan

ARTICLE INFO

Article history:
Received 18 August 2009
Received in revised form 14 December 2009
Accepted 9 March 2010

Keywords:
Amyotrophic lateral sclerosis (ALS)
Cytokines
Chemokines
Cerebrospinal fluid (CSF)

ABSTRACT

We measured the levels of 27 cytokines/chemokines and growth factors in cerebrospinal fluid (CSF) from 42 patients with sporadic amyotrophic lateral sclerosis (ALS), 12 patients with lower motor neuron disease (LMND), and 34 control patients with non-inflammatory neurological diseases (OND), using a multiplexed fluorescent bead-based immunoassay. Among cytokines/chemokines elevated in ALS, CCL2 and CXCL8 levels were negatively correlated with the revised ALS functional rating scale (ALSFRS-R) score, while CCL4 showed a positive correlation with ALSFRS-R score. CCL4 and CXCL10 showed negative correlations with disease progression rate. These chemokine alterations are assumed to somehow correlate with the clinical course of ALS.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease in which loss of motor neurons in the spinal cord, brainstem and motor cortex causes progressive paralysis. Studies using ALS model mice have reported that non-cell-autonomous cell death is a major contributor to motor neuron death (Boillée et al., 2006; Clement et al., 2003; Lobsiger and Cleveland, 2007; Yamanaka et al., 2008). Neuroglial inflammation is thus suggested to be crucial for motor neuron loss. Even in human sporadic ALS, increasing evidence suggests that certain cytokines/chemokines and growth factors, key mediators of both immune and neural networks, play critical roles in certain stages of ALS (Consilvio et al., 2004; McGeer and McGeer, 2002).

In human ALS, the levels of CCL2 (also known as macrophage chemoattractant protein-1) (Henkel et al., 2004; Wilms et al., 2003), interleukin (IL)-6 (Sekizawa et al., 1998), tumor necrosis factor (TNF)-α (Moreau et al., 2005; Poloni et al., 2000), and transforming growth factor (TGF)-β (Ilzecka et al., 2002) have been reported to be elevated in cerebrospinal fluid (CSF). We also measured the levels of 16 cytokines and chemokines in CSF from ALS patients by multiplexed fluorescent bead-based immunoassay, and found that CCL2, IL-5, and granulocyte-colony stimulating factor (G-CSF) are significantly elevated in patients with ALS compared with the levels in patients with non-inflammatory neurologic diseases (Tanaka et al., 2006). Among these, CCL2 showed a significant negative correlation with the revised ALS functional rating scale (ALSFRS-R) score, suggesting the possibility that this chemokine is a disease-aggravating factor (Tanaka et al., 2006). Recently, Mitchell et al (2009) reported that a variety of proinflammatory cytokines and growth factors, namely, CCL2, CCL3 (macrophage inflammatory protein-1α), CCL4 (macrophage inflammatory protein-1β), IL-2, IL-6, IL-15, and IL-17, G-CSF, vascular endothelial growth factor (VEGF), granulocyte-macrophage colony stimulating factor (GM-CSF), and basic fibroblast growth factor (bFGF), were all elevated in ALS patients' CSF. They reported that none of these had any significant correlation with clinical parameters, but that non-elevated CXCL8 (IL-8) had a weak negative correlation with the ALSFRS-R score. No biomarkers related to neuroprotection in ALS are known. Therefore, in the present study, we profiled CSF cytokines/chemokines and growth factors to identify those related to the clinical parameters of ALS and lower motor neuron disease (LMND).

2. Materials and methods

2.1. Patients

A total of 42 patients with sporadic ALS (20 males and 22 females; mean age ± standard deviation [SD] at examination, 56.7 ± 13.2 years) and 12 patients with sporadic LMND (six males and six females; 55.2 ± 15.7 years) were examined (Table 1). All patients with ALS were subjected to a thorough neurological examination and diagnosed as clinically definite or probable cases of ALS based on the El Escorial diagnostic criteria (Brooks, 1994) at the Department of Neurology,

* Corresponding author. Department of Neurology, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan. Tel.: +81 92 642-5337; fax: +81 92 642-5352.
E-mail address: kira@neuro.med.kyushu-u.ac.jp (J. Kira).

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doi:10.1016/j.jneuroim.2010.03.004

Table 1
Demographic features of patients with sporadic amyotrophic lateral sclerosis (ALS), lower motor neuron disease (LMND), and other non-inflammatory neurological diseases (OND).

	ALS	LMND	OND
Number of patients	42	12	34
Sex (male/female)	20/22	6/6	21/13
Age at examination (mean ± SD, years)	56.7 ± 13.2	55.2 ± 15.7	54.2 ± 12.9
Disease duration (mean ± SD, months)	13.0 ± 9.3	25.9 ± 28.6	NA
Immunologic treatment (for the past years)	None	None	None
ALSFRS-R score (mean ± SD)	39.0 ± 8.1	39.6 ± 6.72	NA
CSF			
Cell count (mean ± SD, per μl)	1.15 ± 1.04	1.27 ± 0.78	1.08 ± 1.11
Total protein in CSF (mean ± SD, mg/dl)	34.0 ± 14.3	42.4 ± 27.3	37.3 ± 17.7

Abbreviations in table: ALSFRS-R, revised amyotrophic lateral sclerosis functional rating scale; CSF, cerebrospinal fluid; NA, not applicable; SD, standard deviation.

Kyushu University Hospital, from 2000 to 2006. The mean disease duration at the time of CSF withdrawal was 13.0 ± 9.3 months in ALS patients and 25.9 ± 28.6 months in LMND patients. The disability level associated with the development and progression of ALS and LMND was determined using the revised ALS functional rating scale (ALSFRS-R) (Cedarbaum et al., 1999). The mean ALSFRS-R score was 39.0 ± 8.1 in ALS patients, and 39.6 ± 6.72 in LMND patients. The disease progression rate was defined as ALSFRS-R full score (48) − a patient's ALSFRS-R score/disease duration expressed in months. Thirty-four control patients with other non-inflammatory neurological diseases (OND) but no malignancies (21 males and 13 females; age at examination, 54.2 ± 12.9 years) examined during the same period were also enrolled. The OND group comprised 10 patients with cervical spondylosis, eight with sporadic spinocerebellar degeneration, four with lumbar herniation, four with metabolic neuropathy, two with hereditary spinocerebellar atrophy (SCA3 and unknown), and one each with spastic spinal paraplegia, drug-induced dystonia, peroneal nerve palsy, normal pressure hydrocephalus, Strüthers' ligament syndrome, senile blepharoptosis, and urge incontinence. No subjects were hypoxemic or undergoing any immunotherapies at the time of CSF drawing. The male-to-female ratio was not significantly different among these groups according to the chi-square test ($p>0.1$). We compared the disease duration and the CSF total protein amounts between ALS and LMND patients using the Mann-Whitney U test. The disease duration was significantly longer in LMND patients than ALS patients ($p=0.0149$), probably reflecting a slower disease course in the former, while the total CSF protein levels were not significantly different between the two groups ($p>0.1$).

2.2. Cerebrospinal fluid collection

CSF samples were obtained by lumbar puncture from all patients and immediately centrifuged at 800 rpm at 4 °C for 5 min. The liquid phase of CSF that excluded the sedimented cells was stored at −80 °C until cytokine assay. CSF findings are shown in Table 1. No patients were considered to have systemic inflammation at the time CSF was drawn, because none had elevated serum C-reactive protein level or systemic autoantibodies, such as antinuclear antibody, SS-A and SS-B.

2.3. Multiplexed fluorescent bead-based immunoassay of CSF

The CSF liquid phase samples were simultaneously analyzed for 27 cytokines and chemokines, namely, IL-1β, IL-1 receptor antagonist (IL-1ra), IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12(p70), IL-13, IL-15, IL-17, TNF-α, interferon (IFN)-γ, CCL2, CCL3, CCL4, CCL5 (regulated upon activation normal T-cell expressed and secreted), CCL11, CXCL8, CXCL10, G-CSF, GM-CSF, bFGF, platelet-derived growth factor-bb (PDGFbb), and VEGF, using the Bio-Plex Cytokine Assay System (Bio-Rad Laboratories, Hercules, CA), as described previously (Ishizu et al., 2005; Tanaka et al., 2006). Briefly, 50 μl of each CSF liquid and various concentrations of each cytokine standard (Bio-Rad) were added to 50 μl of antibody-conjugated beads (Bio-Rad) in 96-well filter plates (Millipore, Billerica, MA). Cytokine concentrations were calculated by reference to a standard curve for each cytokine derived using various concentrations of the cytokine standards (0.2, 0.78, 3.13, 12.5, 50, 200, 800 and 3200 pg/ml) assayed in the same manner as the CSF samples. The same batch of monoclonal antibodies for the Bio-Plex Cytokine Assay System was used throughout the experiments; the interassay and intraassay variabilities are reported to be less than 10% by the manufacturer (de Jager et al., 2003; Vignali, 2000). The detection limit for each cytokine was determined by recovery of the corresponding cytokine standard, and the lowest values with more than 70% recovery were set as the lower detection limits. The lower detection limits were as follows: 12.5 pg/ml for GM-CSF and IFN-γ, 3.13 pg/ml for IL-1ra, IL-2, IL-4, IL-6, IL-9, IL-13, IL-17, TNF-α, CCL2, CCL3, CCL11, CXCL10, G-CSF, bFGF, and VEGF, 0.78 pg/ml for IL-12(p70), CCL4, and PDGFbb, and 0.2 pg/ml for IL-1β, IL-5, IL-7, IL-10, IL-15, CCL5, and CXCL8. All samples were analyzed undiluted in duplicate.

2.4. Statistical analyses

We used the following statistical tests for appropriate applications. The non-parametric Kruskal–Wallis *H* test was initially employed to compare the age at CSF withdrawal and CSF cytokine/chemokine levels among the studied group. When differences were significant, the Mann–Whitney *U* test was used to determine the significance of differences between each group. For multiple comparisons, uncorrected *P* values (P^{uncorr}) were corrected by multiplying them by the number of comparisons to calculate corrected *P* values (P^{corr}) (Bonferroni–Dunn's correction). The disease duration and the CSF protein amounts were compared using the Mann–Whitney *U* test. Spearman's rank correlation analysis was used to correlate various clinical parameters and CSF cytokine/chemokine levels which were significantly different among ALS, LMND and control. The male to female ratios were compared among the groups using the chi-square test. Statistical significance was set at $P<0.05$.

3. Results

3.1. Concentrations of each cytokine/chemokine in the liquid phase of CSF

Among the cytokines/chemokines measured, G-CSF, VEGF, CCL2, CCL4, CCL5, CCL11, CXCL8, CXCL10, TNF-α, IFN-γ, IL-1β, IL-7, IL-9, IL-12 (p70), and IL-17 levels were significantly higher in ALS than in OND patients (G-CSF: 9.670 ± 0.484 vs. 7.875 ± 0.537, $P^{corr}=0.0005$; VEGF: 8.450 ± 0.676 vs. 4.855 ± 0.751, $P^{corr}=0.0039$; CCL2: 276.755 ± 11.817 vs. 199.810 ± 13.134, $P^{corr}<0.0001$; CCL4: 12.820 ± 0.974 vs. 7.700 ± 1.082, $P^{corr}=0.0048$; CCL5: 0.845 ± 0.653 vs. 0.300 ± 0.726, $P^{corr}=0.0165$; CCL11: 10.535 ± 0.551 vs. 8.395 ± 0.612 pg/ml, $P^{corr}=0.0072$; CXCL8: 35.040 ± 1.498 vs. 24.335 ± 1.665, $P^{corr}<0.0001$; CXCL10: 456.545 ± 42.442 vs. 289.760 ± 47.171, $P^{corr}<0.0001$; TNF-α: 79.850 ± 3.266 vs. 61.125 ± 3.629, $P^{corr}=0.0031$; IFN-γ: 24.370 ± 1.355 vs. 19.590 ± 1.506, $P^{corr}=0.0132$; IL-1β: 0.955 ± 0.091 vs. 0.685 ± 0.101, $P^{corr}=0.0348$; IL-7: 1.495 ± 0.075 vs. 1.125 ± 0.084, $P^{corr}=0.0135$; IL-9: 27.090 ± 1.074 vs. 20.675 ± 1.193, $P^{corr}=0.0020$; IL-12(p70): 6.900 ± 0.524 vs. 5.065 ± 0.582, $P^{corr}=0.0339$; and IL-17: 2.700 ± 0.194 vs. 2.700 ± 0.215, $P^{corr}=0.0027$) (Fig. 1). The levels of the other cytokines/chemokines did not differ significantly between the two groups. No significant difference was found between the OND and LMND groups in the levels of any of the cytokines/chemokines examined. We found no significant